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Honey bee promoter sequences for targeted gene expression

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Abstract

The honey bee, *Apis mellifera*, displays a rich behavioural repertoire, social organization and caste differentiation, and has an interesting mode of sex determination, but we still know little about its underlying genetic programs. We lack stable transgenic tools in honey bees that would allow genetic control of gene activity in stable transgenic lines. As an initial step towards a transgenic method, we identified promoter sequences in the honey bee that can drive constitutive, tissue-specific and cold shock-induced gene expression. We identified the promoter sequences of *Am-actin5c*, *elp2l*, *Am-hsp83* and *Am-hsp70* and showed that, except for the *elp2l* sequence, the identified sequences were able to drive reporter gene expression in *Sf*21 cells. We further demonstrated through electroporation experiments that the putative neuron-specific *elp2l* promoter sequence can direct gene expression in the honey bee brain. The identification of these promoter sequences is an important initial step in studying the function of genes with transgenic experiments in the honey bee, an organism with a rich set of interesting phenotypes.

Keywords: *Apis mellifera*, honey bee promoters, gene expression, insect cell culture, electroporation.

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Introduction

Honey bees (*Apis mellifera*) are economically important pollinators of wild flowers and crop plants, and they express remarkable features that make the honey bee an interesting model for understanding basic biological phenomena. However, we lack tools to manipulate genes in stable transgenic lines, which would facilitate the dissection of the genetic control of honey bee behaviour and development (Menzel *et al*., 2006). Examples of interesting honey bee features include a rich behavioural repertoire, social organization, caste differentiation and a divergent mode of sex determination. Sex determination in honey bees is controlled by the genotype of a single gene called *complementary sex determiner* (*csd*; Beye *et al*., 2003), which differs from the well-studied sex chromosome systems (Goodfellow & Lovellbadge, 1993; Cline & Meyer, 1996). Females are heterozygous for the *csd* gene, whereas males are homozygous or hemizygous (Hasselmann *et al*., 2008; Gempe *et al*., 2009), but the basis of sexual differentiation is only partially understood (Gempe & Beye, 2011).

A honey bee colony consists of thousands of essentially sterile female workers and one single fertile female, the queen (Winston, 1987), that lays up to 1500 eggs per day (Gary, 1992; Katzav-Gozansky *et al*., 2001). This pronounced caste differentiation relies on differential nutrition with royal jelly (Haydak, 1970; Kamakura, 2011) and epigenetic control of gene activity (Kucharski *et al*., 2008). Honey bee workers perform a rich and diverse repertoire of behavioural tasks that help maintain the colony (von Frisch, 1967; Winston, 1987; Seeley, 1995). The behavioural activities of workers are performed in highly coordinated ways and involve task specialization and division of labour (Robinson, 1992; Page & Erber, 2002). Workers also show impressive cognitive abilities; for instance, they can learn abstract concepts, such as ‘same’ and ‘different’ (Giurfa *et al*., 2001). Furthermore, workers can communicate the location of food sources to other colony members of the hive via waggle dances (von Frisch, 1967).

In the present study, we identified for the first time honey bee promoter sequences that are suitable for driving

transgenic transcription. Targeted gene expression is an important tool for basic and applied research in the honey bee. In a previous study, Kunieda & Kubo (2004) were able to drive the expression of the enhanced green fluorescent protein (EGFP) reporter gene in the honey bee brain by the human cytomegalovirus (CMV) promoter. They introduced the respective plasmid into honey bee nervous cells by electroporation. Transgenic studies in the fruit fly *Drosophila melanogaster*, the medfly *Ceratitis capitata* and the red flour beetle *Tribolium castaneum* showed that endogenous promoters are essential tools for controlling gene expression in the respective species (Kalosaka *et al*., 2006; Schinko *et al*., 2010). We followed this approach and tried to identify honey bee promoter sequences that would promote transcription in neuronal tissue in the entire organism and only after temperature-dependent induction. We used the genetic information of orthologues in *D. melanogaster*, whose promoters are widely used in transgenic experiments, to identify putative candidate promoters. In *D. melanogaster*, the *actin5c (act5c)* gene is ubiquitously expressed throughout development (Fyrberg *et al*., 1983), and its promoter sequence has been used to drive the overexpression of transgenes (Chung & Keller, 1990; Chavous *et al*., 2001). The expression of the *embryonic lethal abnormal vision* (*elav*) gene in *D. melanogaster* is restricted to neuronal tissues (Campos *et al*., 1987; Yao & White, 1994), and its promoter sequence has been widely used to direct expression genetically to the fly brain (Robinson *et al*., 2002; Wu *et al*., 2011). Gene transcription of heat shock proteins (hsps) can increase by 10- to 1000-fold after stress conditions, such as heat shock (Lindquist, 1984; Lindquist, 1986). The *Dm-hsp70* promoter sequence has been widely used to induce knockdown of genes or express cDNA at specific development stages by shifts in the environmental temperature (Monsma *et al*., 1988; Grotewiel *et al*., 1998; Lam & Thummel, 2000; Cheng *et al*., 2001). Promoters of *hsp* genes have also been successfully used in other species, including the lepidopteran insects *Bicyclus anynana* and *Bombyx mori* (Monsma *et al*., 1988; Uhlirova *et al*., 2002; Ramos *et al*., 2006; Schinko *et al*., 2010).

In the present study, we cloned the honey bee orthologues of these genes, which are candidates for these tissue-specific promoters, general and inducible promoter sequences, and we studied whether they can induce expression of the reporter gene *rubia* in *Sf*21 insect cells or in the honey bee brain. Our attempts to study the expression of our reporter gene constructs directly in honey bees failed, regardless of whether we transferred the plasmids via injection (Beye *et al*., 2002) or via sperm (Robinson *et al*., 2000) to the embryos (information on this nonfunctional pilot study is given in supporting information SI1). Here, we report on the first honey bee candidate promoter sequences that may be suitable for directing the expression of genes in nervous tissue, the entire organism and at all developmental stages after a temperature shift.

Results

Honey bee-derived promoter sequences direct gene expression in Sf21 *cells*

We amplified putative promoter sequences of *Am-actin5c*, *elp2l*, *Am-hsp70* and *Am-hsp83* gene from the honey bee genome using PCR (Fig. 1). We cloned the fragments into the pIZ/V5-His plasmid (Invitrogen, Darmstadt, Germany) upstream of the translational start codon of the reporter gene, *rubia*. We amplified a minimum of 1000 bp upstream of the translation start site of the corresponding gene which included the entire 5′ untranslated region (5′UTR). We identified 5′ ends of transcripts by reverse transcriptase PCR (RT-PCR) experiments to assign putative transcriptional start points. Our putative promoter sequence of the *actin5c* gene of *Apis mellifera* (GB12453)

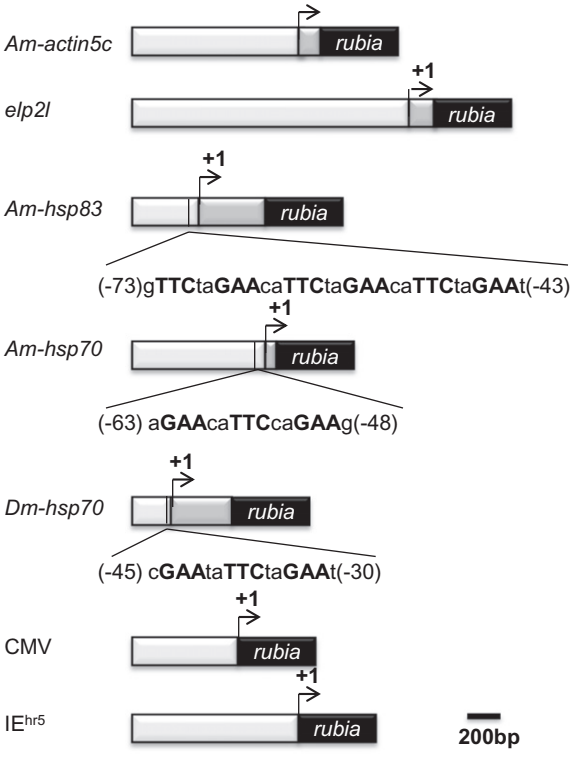

Figure 1. Presentation of the expression cassettes that were cloned into the pIZ/V5-His plasmid. Light grey boxes indicate the genomic region upstream of the transcription start; dark grey boxes show the 5′ untranslated region. Black boxes indicate the region encoding for the red fluorescence protein Rubia. The scale indicates the sizes of the boxes (bp). The position of the heat shock element sequence motifs and their position upstream of the respective transcription start sites is indicated below the boxes; +1 denotes the transcription start site.

encompasses a 1420-bp fragment upstream of the translation start site, including the entire 5′ UTR (Fig. 1). The *Am-actin5c* gene is the orthologue of the *actin5c* gene of *D. melanogaster*. The fruit fly gene is expressed throughout development (Chung & Keller, 1990), and its promoter sequence has been used to constitutively express transgenes (Chavous *et al*., 2001). The putative promoter sequence of the *elp2l* gene (GB18785) encompasses 2000 bp upstream of the translation start with 207 bp of that being the 5′UTR. The *elp2l* gene is annotated in the National Center for Biotechnology Information (NCBI) database as a homologue of the *elav* gene of *D. melanogaster* (further information on homology is shown in SI2). The *elav* promoter sequence has been widely used in transgenic fruit flies to drive the neuronal expression of genes (Samson & Chalvet, 2003; Wu *et al*., 2011). The putative promoter sequence of the *Am-hsp70* gene (GB14852), including the 5′UTR, encompasses 1001 bp upstream of the translation start codon. The expression of the *Am-hsp70* gene increased after heat shock in foraging honey bee workers (Elekonich, 2009), suggesting that the promoter sequence can drive expression after a heat shock. The putative promoter sequence of the *Am-hsp83* gene (GB14494) encompasses 1000 bp sequence upstream of the translational start codon, including a 490 bp 5′UTR. We suggest the promoter of *Am-hsp83* gene is a good candidate for driving temperature shift-inducible transcription as the transcription of the orthologue gene *Se-hsp83* in the lepidopteran insect *Spodoptera exigua* was inducible by cold shock (Xu *et al*., 2011). We listed the sequences of the putative promoters in the supplementary information (SI3).

We checked for conserved heat shock elements (HSEs) in the sequences of our putative *Am-hsp70* and *Am-hsp83* promoter sequences. These elements consist of a highly conserved nGAAn sequence or the corresponding palindromic sequence nTTCn that are present in different copy numbers and are required to bind the heat shock factors that promote transcription of the heat shock protein genes (Pirkkala *et al*., 2001; Sakurai & Enoki, 2010). We found six HSEs 43–73 bp upstream of the transcription start site in the *Am-hsp83* sequence (Fig. 1). In the *Am-hsp70* sequence, we identified three HSEs 48–63 bp upstream of the transcription start site (Fig. 1). The presence of these reiterated motifs in the putative *Am-hsp83* and *Am-hsp70* promoter sequences suggests a possible role for these sequences in driving expression after temperature shock. We compared the gene expression function of the honey bee sequences in subsequent experiments with: the already characterized *hsp70* promoter of *D. melanogaster* (Pelham, 1982) which has been widely used as an inducible promoter in transgenic flies (Monsma *et al*., 1988); the human CMV promoter (Sinclair, 1987; Kunieda & Kubo, 2004); and the baculovirus-derived OplE2 and IE^{hr5} promoters (Granados & Federici, 1986; Theilmann & Stewart, 1992; Cartier *et al*., 1994).

We transfected 2.5 μg of the respective plasmids into 10^6 *Sf*21 cells using Roti® Insectofect reagent (Roth, Karlsruhe, Germany). We studied the *Sf*21 cells 48 h after transfection to determine whether they showed red fluorescence signals compared with non-transfected control cells. In *Sf*21 cells that were transfected with plasmids containing the *Am-actin5c, Am-hsp70* or *Am-hsp83* putative promoter sequences, we observed red fluorescence compared with untransfected cells (Fig. 2A,C,D,J), suggesting that the cloned honey bee sequences can drive expression of the Rubia protein in *Sf*21 cells. We also observed red fluorescence in cells that were transfected with vectors containing the *Dm-hsp70*, the OplE2, the IE^{hr5} and the CMV promoter sequences (Fig. 2E–H). Cells transfected with the plasmid containing the putative *elp2l* promoter sequence displayed no fluorescence (Fig. 2B), suggesting that the Rubia protein was not expressed. This result shows that the *elp2l* promoter, which is supposed to drive expression in the neuronal substrate in honey bees, did not drive expression in *Sf*21 cells that were derived from ovarian tissues. This negative result, however, indicates that the core promoter sequence alone is not sufficient to drive expression of the reporter gene in our assay.

We compared the relative strength of the promoter activity by semi-quantifying the fluorescence intensity of single cells. Because the fluorescence intensities of transfected cells are strongly affected by the stage of the cell's cycle, we chose the three brightest cells for each picture. These cells should have represented cell samples that were presumably at similar cell-cycle stages and this procedure allowed us to approximate fluorescence intensities and therefore relative expression levels for each promoter. We analysed 10 independent transfection experiments for each promoter. For each transfection we imaged a set of cells (we detected a mean (SEM) cell number of 134 (± 44) cells and 17(± 7) transfected cells per picture; supporting information SI4 shows the number of detected cells for each promoter construct) and measured the mean light intensity values for each of the three brightest cells in the picture. We used the same excitation and detection settings on the microscope, with the relative light intensities ranging from 0 to 250.

We found the following order of expression according to the arbitrary fluorescence intensities (high to low): OplE2, *Dm-hsp70*, IE^{hr5}, *Am-hsp83*, *Am-actin5c*, CMV and *Am-hsp70* (Table 1). This result indicates that the honey bee-derived promoter sequences induce lower levels of expression than the promoters that are derived from the baculovirus (OplE2, IE$^{hr5)}$). These viral promoters are strong promoters and are highly adapted to the lepidopteran *Sf*21 cells (Theilmann & Stewart, 1992; Cartier *et al*., 1994).

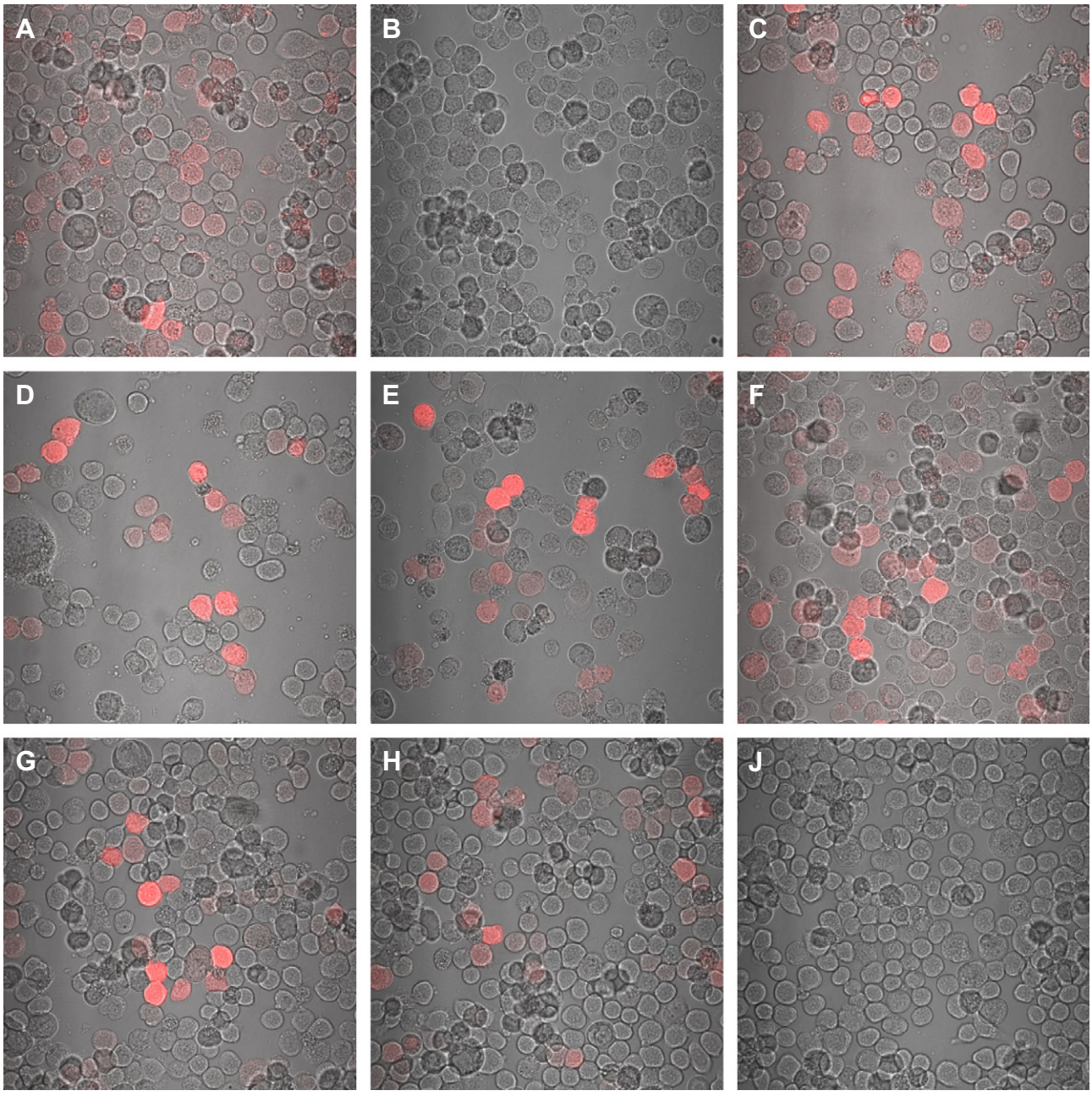

Figure 2. Expression of Rubia protein driven by the putative promoter sequences in *Sf*21 cells. The images are presented as overlays of the red fluorescence detection picture and the bright light picture, which were taken by a confocal microscope. A-H: 10^6 cells were transfected with 2.5 µg of the pIZ/V5 plasmid driving Rubia protein expression with the indicated putative promoter sequences. A: *Am-actin5c*; B: *elp2l*; C: *Am-hsp70*; D: *Am-hsp83*; E: *Dm-hsp70*; F: OplE2; G: cytomegalovirus; H: IE^{hr5}; J: control, untransfected *Sf*21 cells.

The Am-hsp83 *promoter-derived expression is enhanced by temperature shift*

We studied whether the expression level of the *rubia* reporter gene driven by the *Am-hsp70*, *Am-hsp83* and *Dm-hsp70* promoters can be enhanced by shifting the constant incubating temperature of 27 °C to higher (34 °C) or lower (16 °C) temperatures for 1.5 h. From one transfection experiment we split the transfected cells and studied the effect of temperature shifts and non-shifts on expression in independent cell samples. We did not study the same cell samples before and after the temperature shifts because the microscopical observations and microbial infection of the cell culture (as a result of these observations) could both stress the cells and could possibly induce expression that is independent of our treatment

Table 1. The relative strength of the honey bee promoter sequences in *Sf*21 cells as measured by fluorescence intensities of Rubia proteins

Promoter	Expression strength
Am-actin5c	++
elp2l	–
Am-hsp70	+
Am-hsp83	+++
Dm-hsp70	+++++
OplE2	+++++
Cytomegalovirus	++
IE^{hr5}	++++
Untransfected	–

Red fluorescence intensities were measured from the picture data. The three brightest cells per picture for each promoter sequence were counted. We calculated the mean fluorescence intensity values from 10 independent transfection experiments. We ordered the values of the fluorescence intensities from '+++++' (OplE2) to '–' (untransfected).

(the temperature shifts). We first determined whether the fluorescence intensities of the untransfected *Sf*21 cells were affected by temperature shifts, but we did not observe any effect (data not shown).

To estimate the relative increase in fluorescence after the temperature shifts, we obtained a fluorescence threshold under non-shifted conditions (constant temperature of 27 °C). We took four pictures of different cells [mean (± SEM) number of detected cells per picture 145 (± 58)] for each transfection in which the temperature was non-shifted and set the threshold above the brightest fluorescent cell for each picture. From these four thresholds, we built a mean threshold for the respective transfection. We shifted the temperature of the independent cell samples (but derived from the same transfection) to 16 and 34 °C for 1.5 h, and determined the number of cells above that threshold (Figs 3 and 4) that we obtained from the non-shifted cell samples. If more fluorescent cells over the threshold were detected after a temperature shift this was taken as evidence of an increase in expression attributable to the temperature shift. To determine these numbers, we took four pictures of different cells for each transfection. Each of these pictures (shifted and non-shifted cells) comprises a mean (± SEM) number of 145 (± 58) cells and 10 (± 6) transfected cells (SI5 shows the

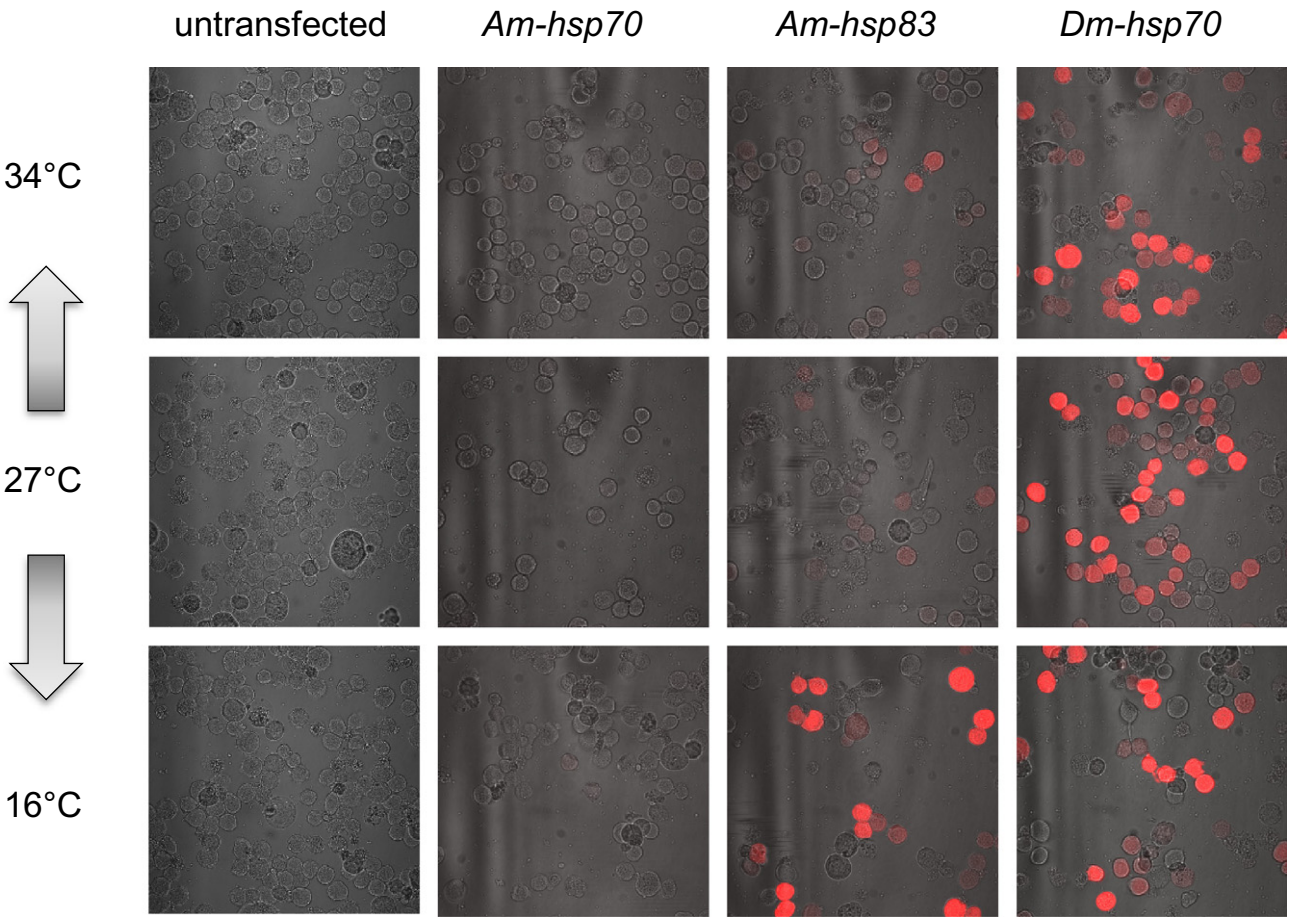

Figure 3. The effects of temperature shifts on *hsp* promoter-derived expression of Rubia protein in *Sf*21 cells. Temperature shifts were performed for 1.5 h 48 h after transfection. Heat shock was performed at 34 °C; cold shock was performed at 16 °C.

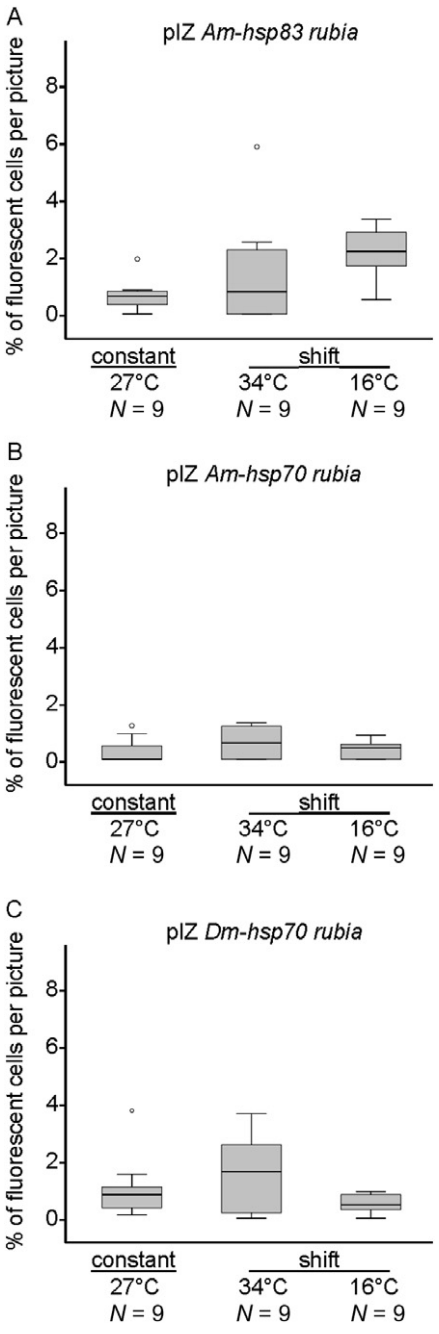

Figure 4. Box plots displaying the relative number of fluorescent cells above the threshold in response to the different temperature treatments. The threshold was set such that the fluorescence of the transfected cells that were held under constant temperature conditions should show no fluorescent signal (see methods). Cells were transfected with 2.5 μg plasmids that have the promoter sequences of the given gene. A: *Am-hsp70*; B: *Am-hsp83*; C: *Dm-hsp70*. The relative numbers of fluorescent cells between the temperature treatments were compared using the Mann–Whitney *U*-test; *** denotes $P < 0.001$.

detailed numbers of cells for each promoter construct). In all, we studied construct cells from nine different transfection experiments for each promoter.

For the *Am-hsp83* promoter sequence, the number of fluorescent cells above the threshold increased almost threefold after 16 °C treatment (Mann–Whitney *U*-test; $P < 0.001$; Figs 3 and 4A) compared with the number of cells above the threshold in the constant temperature conditions. Temperature shifts to 34 °C did not increase the number of fluorescent cells ($P > 0.05$; Figs 3 and 4A). We conclude that shifting the temperature to 16 °C increases the *Am-hsp83* promoter-driven expression of *Rubia* protein in *Sf*21 cells. Shifting the cells containing the *Am-hsp70* or the *Dm-hsp70* promoter sequence to 16 °C or to 34 °C did not increase the number of fluorescent cells above the threshold compared with the cells held at a constant temperature (Mann–Whitney *U*-test, $P > 0.05$; Figs 3 and 4B,C). The latter result suggests that temperature shifts were not able to enhance the protein expression of genes regulated by the *Am-hsp70* or *Dm-hsp70* promoter sequences in *Sf*21 cells.

The elp2l *promoter sequence can direct gene expression in the honey bee brain*

Our study showed that the *elp2l* promoter sequence is not able to drive expression of the Rubia protein in *Sf*21 cells, which are ovary-derived (Fig. 2E). A first survey of transcription in different honey bee tissues via RT-PCR led to a proposed confined expression of the *elp2l* honey bee gene in neuronal tissue (Schulte, pers. comm.). To further test, whether the putative *elp2l* promoter sequence can direct expression in honey bee nervous tissue, we introduced the pIZ/V5 *elp2l* EGFP plasmid into honey bee brains via electroporation. This pIZ/V5 *elp2l* EGFP plasmid consists of the *elp2l* promoter sequence, which was cloned in front of the EGFP reporter gene. The electroporation method for honey bees was devised by Kunieda & Kubo (2004) to introduce DNA into the honey bee brain. We injected 520 ng of the respective plasmid into each brain via the middle ocellus and electroporated them afterwards. In addition to the pIZ/V5 *elp2l* EGFP plasmid, we also electroporated the peGFP-C1 plasmid that contains a CMV promoter, which has been previously shown to drive EGFP protein expression in the honey bee brain (Kunieda & Kubo, 2004). We also electroporated with the pBluebac plasmid, which comprises the *polyhedrin* gene promoter sequence from baculovirus and comprised no reporter gene. We used this plasmid as a negative control to avoid non-specific effects from the electroporation procedure. Three days after the electroporation (58.6% of the bees survived), we immunostained the brains and detected the EGFP protein via mouse anti-EGFP antibody and a secondary goat anti-mouse

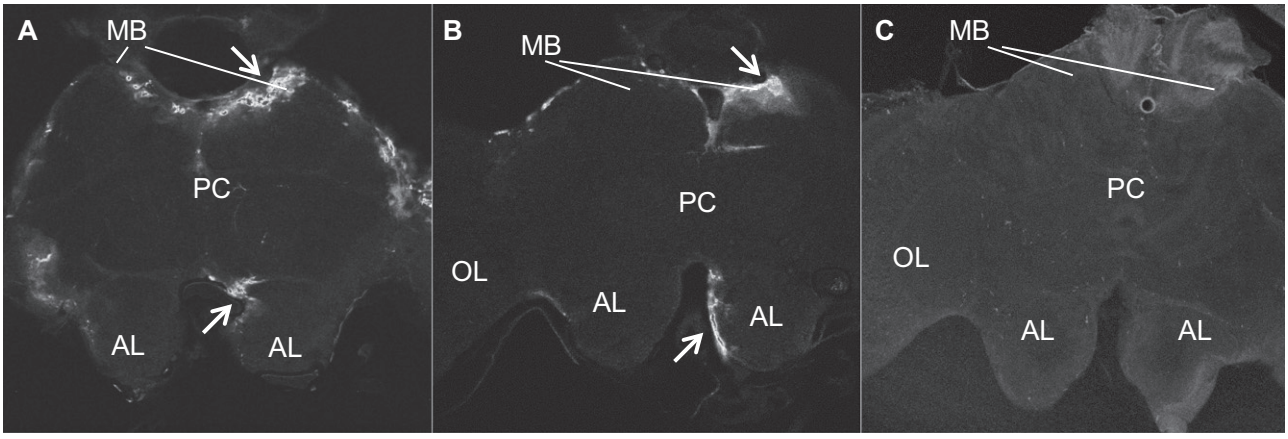

Figure 5. Immunostaining of EGFP protein expression after electroporation of plasmid DNA into the honey bee brain. Examples of the immunostaining of EGFP protein with mouse-derived anti-EGFP antibody that was detected with a Cy5-conjugated goat anti-mouse antibody. A: peGFP-C1; B: pIZ/V5 *elp2l* EGFP; C: pBluebac; AL: antennal lobe; PC: protocerebrum; MB: mushroom bodies; OP: optical lobes; arrows indicate regions of strong Cy5 fluorescence signal denoting EGFP expression.

antibody, which is conjugated with the fluorophore Cy5. Electroporation with the pIZ/V5 *elp2l* EGFP plasmid produced Cy5-fluorescence signals in distinct parts of the brain, indicating that the EGFP protein is expressed ($n = 4$; Fig. 5A). The expression is found in peripheral parts of the protocerebrum, the antennal lobes and the mushroom bodies. When we electroporated brains with peGFP-C1, which served as a positive control (Kunieda & Kubo, 2004), we detected a similar Cy5-fluorescence pattern in the brain as with our pIZ/V5 *elp2l* EGFP plasmid ($n = 3$; Fig. 5B). This result suggests that the CMV and the *elp2l* promoter produce similar expression patterns only in the peripheral parts of the brain, most likely owing to the lack of entire spreading of the DNA during the electroporation procedure. In brains which we electroporated with the pBluebac plasmid ($n = 5$; Fig. 5C), we detected no fluorescence, indicating that our method produced no artificial Cy5-fluorescence signal. Taken together, these results show that our honey bee-derived *elp2l* promoter sequence can direct expression of genes in the honey bee brain.

Discussion

In this study, we cloned several general, tissue-specific and inducible candidate promoter sequences from the honey bee (*A. mellifera*) and showed that the general (*Am-actin5c*) and inducible promoters (*Am-hsp70* and *Am-hsp83*) can drive expression in lepidopteran-derived *Sf*21 cells. We also showed that the *Am-hsp83* is a cold shock-inducible promoter in *Sf*21 cells. Because we lack transgenic methods in honey bees, we took advantage of the *Sf*21 cell system. If the candidate promoters from the honey bee genome can induce expression in the heterologous system of *Sf*21 cells, we took it as a first hint that they may also drive expression in honey bees. We also identified a candidate of tissue-specific promoter that can drive expression in nervous cells of the honey bee brain but not in *Sf*21 cells. Hence, we identified candidate honey bee promoters that can drive ubiquitous, nervous tissue-specific and inducible expression of transgenes in the honey bee.

We showed that the *Am-actin5c* promoter sequence can drive gene expression in *Sf*21 cells, suggesting that this sequence harbours the relevant sequence information required to promote transcription in this cell line. In *Sf*21 cells, the *Am-actin5c*-derived expression is weak compared with viral promoters. In *D. melanogaster*, the endogenous promoter sequence of the *actin5c* gene has been widely used to drive constitutive overexpression of transgenes (Bond & Davidson, 1986; Thummel *et al.*, 1988). We thus also suggest that the *Am-actin5c* promoter sequence can be used in an equivalent way to promote constitutive expression of transgenes in the honey bee.

We showed that the putative *elp2l* promoter cloned fragment could not drive gene expression in *Sf*21 cells. This result suggests that the general core promoter sequence that is located directly upstream of the transcription start site and contains the putative TATA-Box and the putative initiator sequence is not sufficient to drive gene expression in the *Sf*21 cells. This negative result shows that the core promoter sequence alone is not sufficient to direct expression of the reporter gene in *Sf*21 cells. We electroporated the *elp2l* promoter sequence into the brain of honey bees and showed that this sequence can drive gene expression in the nervous tissue. We suggest that activation of the *elp2l* promoter requires some species- or tissue-specific factors (the *Sf*21 cells are derived from ovarian cells) to promote transcription. The promoter

sequence of the *D. melanogaster elav* gene, which is a homologue of the *elp2l* gene, has been widely used to express transgenes in neuronal tissues (Yao & White, 1994; McGuire *et al*., 2004). For instance, Wu *et al*. (2011) rescued *D. melanogaster* mutants of neuronal receptors PlexB by expressing the *plexB* gene under the control of the *elav* promoter in all neurons. The *elav* gene of *D. melanogaster* has two paralogous copies, the *found in neurons* (*fne*) and the *riboprotein 9* (*rbp9*) genes (Samson & Chalvet, 2003), whereas the *elp2l* gene of the honey bee is a single copy gene, that expresses in neuronal tissues, but not in muscle tissues (Schulte, pers. comm.). All of the fruit fly paralogous genes are expressed solely in neuronal tissues. The Elav protein is present in all neurons throughout development (Robinow & White, 1991), whereas proteins from the *fne* gene were found in neurons of the central nervous system and in the peripheral nervous system during embryogenesis (Samson & Chalvet, 2003) of the fruit fly. *rbp9* is exclusively expressed in neurons of the CNS in *D. melanogaster* (Kim & Baker, 1993). From our experimental evidence, we suggest that the *elp2l* promoter can direct expression of transgenes in the honey bee brain.

We also identified *Am-hsp83* as a cold shock inducible promoter in *Sf*21 cells. A heat shock at 34 °C did not increase the level of expression in *Sf*21 cells. A previous study that measured the level of *Am*-Hsp83 protein expression in the honey bee showed that the *Am-hsp83* promoter is also inducible by heat shock (Chacon-Almeida *et al*., 2000). Chacon-Almeida *et al*. (2000) showed that honey bee workers treated for 4 h with 42 °C showed a higher level of *Am*-Hsp83 protein expression than control worker bees, which were maintained at 34 °C. We suggest that in our study, the temperature for heat shock was not high enough or that the *Sf*21 cell system cannot mimic the cellular heat shock conditions of honey bees. Consistent with our results, the *Se-hsp83* gene in *Spodoptera exigua*, a lepidopteran insect, also increased the level of transcription after cold shock (Xu *et al*., 2011). We suggest, therefore, that we have identified a promoter sequence of the honey bee that can induce higher levels of expression after a temperature shift, *Am-hsp83*.

The *Am-hsp70* promoter was not inducible after temperature shifts from 27 °C to higher (34 °C) or to lower (16 °C) temperatures; however, Elekonich (2009) showed that the amount of *Am*-Hsp70 protein in the heads of worker honey bees increases after shifting the temperature from 33 to 42 °C or 46 °C for 4 h, suggesting that the *Am-hsp70* gene is a heat-inducible gene, which is consistent with our identification of three HSEs in the cloned fragment. A similar motif consisting of three HSEs is present as well in the promoter sequence of the *D. melanogaster hsp70* gene, a motif that is required for temperature shift-induced expression in that species (Pelham, 1982). The HSEs indicate in general a temperature-associated expression pattern of the downstream gene (Lindquist, 1986; Sakurai & Enoki, 2010); therefore, we speculate that we have cloned the *Am-hsp70* promoter sequence but that the temperature shift we applied was not sufficient or that the *Am-hsp70* promoter requires cellular conditions of the honey bee that we cannot mimic in *Sf*21 cells. Nevertheless, the possibility that we missed regulatory regions of the promoter that are important to control the heat shock induced expression cannot be excluded. The *Am-hsp83* promoter sequence contains a HSE motif consisting of six HSEs, whereas the *Am-hsp70* promoter sequence only contains three HSEs. This finding supports our finding that the activity of *Am-hsp83* promoter can be increased during a temperature shift whereas the activity of *Am-hso70* promoter cannot be increased under the same conditions.

Transgenic studies in the fruit fly *D. melanogaster*, the medfly *C. capitata* and the beetle *T. castaneum* showed that endogenous promoters are essential tools for controlling gene expression in the respective species (Kalosaka *et al*., 2006; Schinko *et al*., 2010). Overexpression and ectopic expression of genes and knockdown studies that were mediated by endogenous promoters have been very informative for gaining insights into the underlying genetic programs in *D. melanogaster* (McGuire *et al*., 2004). The honey bee promoter sequences that we identified here are thus valuable genetic tools for controlling the expression of transgenes in the honey bee. A transgenic method has yet to be established in honey bees, but stable transformants mediated by transposons have been successfully applied in different insect species (O'Brochta and Atkinson, 1996). *PiggyBac* and *Minos* are some of the more widely used transposons. Both transposons are absent in the honey bee genome and are good candidates for producing transgenic honey bees. The *piggyBac*-mediated transposition of transgenes has been used in at least 13 insect species that belong to three insect orders [Lepidoptera, Diptera and Coleoptera; reviewed in Handler, (2002)]. *Minos*-derived transpositions have been used in a wide range of species, for instance, in the mosquito *Anopheles stephensi* (Catteruccia *et al*., 2000), in the coleopteran *T. castaneum* (Pavlopoulos *et al*., 2004) or in the medfly *C. capitata* (Zwiebel *et al*., 1995) and even outside the insect class in the crustacean *Parhyale hawaiensis* (Loukeris *et al*., 1995; Catteruccia *et al*., 2000; Pavlopoulos *et al*., 2004).

The control of transgene expression by endogenous promoters will greatly facilitate the genetic analysis of the rich behavioural repertoire, the social organization, caste differentiation and sex determination of the honey bee. The *Am-actin5c* promoter is a candidate for driving ubiquitous expression, while the e*lp2l* promoter can promote expression in the brain. The *elp2l* promoter may

thus enable us to study genes that specify the neuronal substrate that is orchestrating the rich behavioural repertoire of the honey bee. The *Am-hsp83* promoter will allow us to direct the expression or gene knockdowns by temperature shifts, which will enable us to study the function of genes at defined developmental stages.

Taken together, the identification of the first honey bee promoter sequences is an initial and important step in studying the function of genes by transgenic tools in the brain or at different developmental stages in the honey bee, an organism that displays a rich set of interesting and unexplored phenotypes.

Experimental procedures

Cloning of putative promoter sequences

We amplified the genomic region upstream of the corresponding translation start site of the genes *Am-actin5c*, *Am-hsp70* and *Am-hsp83* by PCR from honey bee genomic DNA and the *Dm-hsp70* gene from *D. melanogaster* (Pelham, 1982) genomic DNA. The putative transcription start sites were revealing the 5′end of the transcript via rapid amplification of cDNA ends (RACE) experiments (FirstChoice® RLM-RACE kit, Ambion, Huntington, UK) and RT-PCRs. All the oligonucleotides that were used in this study are listed in the supplementary information (SI6). The sequence information for the amplified genes was obtained from NCBI. We cloned these amplicons into the pIZ/V5 His plasmid (Invitrogen) using the *Sac*I/*Xba*I or the *Hind*III/*Xba*I restriction sites and thereby replaced the OpIE2 promoter sequence just upstream of the pUC ori site. To produce the *elp2l* putative promoter sequence with the 5′UTR, we first amplified the 2000 bp upstream of the transcriptional start site from genomic DNA by PCR. We cloned this fragment into the pGEM-T plasmid (Promega, Mannheim, Germany) using the *Hind*III and *Xba*I restriction sites. This produced the plasmid pGEM-T_fne_prom, in which a new restriction site (*BsaI*) was introduced by an oligonucleotide in the PCR. In the second step, we amplified the 5′UTR region of the *elp2l* gene from honey bee cDNA. We cloned this fragment into the plasmid pGEM-T_fne_prom downstream of the transcription start by using the *BsaI/XbaI* restriction sites. Next, we cloned this entire *elp2l*-derived sequence into the pIZ/V5 His plasmid by using the *Hind*III/*Xba*I restriction sites. We amplified the CMV promoter sequence from the pEPI-EGFP plasmid that was kindly provided by Prof. Hans J. Lipps, University of Witten (Sinclair, 1987; Kunieda & Kubo, 2004; Stehle *et al*., 2007). The IE^{hr5} promoter sequence was amplified by PCR from the plasmid LA928, which was kindly provided by Dr Luke Alphey, University of Oxford (Granados & Federici, 1986; Gong *et al*., 2005). The amplified CMV and IE^{hr5} promoter sequences were cloned into the pIZ/V5 His plasmid using the *Hind*III/*Xba*I restriction sites. We used the pIZ/V5 OpIE2 plasmid from Invitrogen (Theilmann & Stewart, 1992). We inserted the *rubia* reporter gene expressing a red fluorescence protein downstream of the 5′UTRs by using the *Xba*I and *Sac*II restriction sites. To construct the pIZ/V5 *elp2l* EGFP plasmid, we replaced *rubia* with the *egfp* gene using the *Xba*I/*Sac*II restriction sites. The *egfp* gene was amplified by PCR. We used the peGFP-C1 plasmid from Clontech (Saint-Germain-en-Laye, France) and the pBluebac plasmid from Invitrogen.

Cell culture

*Sf*21 cells (Biochrom, Berlin, Germany) were kept adherent at 27 °C in six-well plates and maintained according to the manufacturer's instructions. We transfected 2.5 μg of plasmid DNA into 10^6 cells using Roti Insectofect reagent (Roth).

Expression analysis in Sf21 *cells*

We imaged each of the 10 wells representing independent transfection experiments for each construct. These images were taken under constant excitation and fluorescence detection settings to semi-quantify the relative expression of Rubia proteins for each of the putative promoter sequences. We measured the light intensities from the three most luminous cells of each picture ($N = 10$). We dragged a line across each of the three most luminous cells in each picture, which produced an intensity profile in the LSM 510 Meta software. We estimated the mean values across the cells and pictures that resulted in relative intensity values (ranging from 0 to 250) for each promoter. Red fluorescence was detected by a confocal laser microscope (Zeiss LSM 510 Meta) at a wavelength of 561 nm 48 h after the transfection with the plasmids.

Expression analysis under temperature shift conditions

*Sf*21 cells were transfected with the plasmids containing either the *Am-hsp70*, *Am-hsp83* or *Dm-hsp70* promoter sequences and were kept at 27 °C for 48 h prior to the temperature shift experiments. We either heated (34 °C) or cooled (16 °C) the *Sf*21 cells for 1.5 h. Immediately afterwards, we took four non-overlapping pictures of the cells in each well. To estimate the relative increase in fluorescence after the temperature shifts, we counted the number of cells above a fluorescence threshold. We took four non-overlapping pictures of the non-shifted transfected cells for each transfection and set the threshold above the brightest fluorescent cell in each picture. Based on these four thresholds, we generated a mean threshold for the respective transfection experiment. We counted the number of fluorescent cells above this mean threshold for cells that were treated with higher or lower temperatures and those that were not. We calculated the relative proportion of fluorescent cells above the threshold to all of the cells that were present in the picture that we detected by white light settings. We compared the relative number of fluorescent cells under the shifted and non-shifted temperature conditions using the Mann–Whitney *U*-test, which we conducted in IBM SPSS Statistics (20.0.0).

Electroporation

Adult worker honey bees were caught, anaesthetized on ice and immobilized by putting them in a small plastic tube. A total of 260 nl of the peGFP-C1, pBluebac and pIZ/V5 *elp2l* EGFP plasmids were injected into the median ocelle of a worker honey bee using the method of Mussig *et al*. (2010), at a concentration of 2 μg/μl. Teflon-insulated platinum wire electrodes (diameter 0.125 mm) were used. The insulation was removed at the tip of the electrodes and they were flattened by maintaining them between two metal plates and hitting them with a hammer. The electrodes were introduced in the complex eye, through a cut performed in the dorsal part of the complex eye. The electroporation of the brain was performed with five pulses for 25 ms at

100 V using a CUYEDIT 21 electroporator (Nepagene, Ichikawa, Japan). The mortality rate was of 41.4% (58/140 animals). The brains of the surviving bees were dissected 3 days after electroporation, fixed, dehydrated and cut into 100-μm slices. They were stained with a mouse-derived antibody against GFP (mAB 3E6, Invitrogen) and with a CY5-conjugated secondary antibody (goat anti-mouse, Jackson immunoresearch, Suffolk, UK) and imaged with the Leica TCS-SP2 confocal microscope using a 10× dry objective (HC PL APO CS 10 × 0.4, Leica, Bensheim, Germany). The Helium Neon 633 nm laser line was used to detect the Cy-5 signal (emission 670 nm). All brains were imaged at different laser powers: Fig. 5A: laser power 50%, photomultiplier tube (PMT) 580; Fig. 5B: laser power 70%, PMT 580; Fig. 5C: laser power 94%, PMT 651; thus, the negative control brain was scanned at the highest intensity. Since we were interested only in detecting the GFP signal and not in quantifying them, we did not normalize the positive brains to controls. In all pictures we used a pixel resolution of 1024 × 1024 in *xy* axis and an 8-bit intensity resolution. The voxel size was 0.73 μm × 0.73 μm in Fig. 5A and 1.46 μm × 1.46 μm in Fig. 5B,C.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supporting information SI1. Sperm-mediated or microinjected plasmid transfer into honey bee embryos: pilot experiments.

Supporting information SI2. Blastp search of *D. melanogaster* Elav protein amino acid sequence against the honey bee protein database at the National Center for Biotechnology Information.

Supporting information SI3. Sequences with the designated promoter regions.

Supporting information SI4. The mean numbers of *Sf*21-cells and fluorescent *Sf*21-cells per picture that were used to study the promoter activity. The numbers were estimated from pictures taken from 10 transfections for each promoter construct.

Supporting information SI5. The mean numbers of *Sf*21-cells per picture detected in pictures that were used to study the impact of temperature shifts.

Supporting information SI6. Oligonucleotides that were used in PCRs.